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# Disseminated Acanthamoeba sp. infection in a dog

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### **Abstract**

Several species of free-living amoebae can cause encephalomyelitis in animals and humans. Disseminated acanthamoebiasis was diagnosed in pyogranulomatous lesions in brain, thyroid, pancreas, heart, lymph nodes, and kidney of a one-year-old dog. *Acanthamoeba* sp. was identified in canine tissues by conventional histology, by immunofluorescence, by cultivation of the parasite from the brain of the dog that had been stored at  $-70\,^{\circ}$ C for two months, and by PCR. The sequence obtained from the PCR product from the amoeba from the dog was compared to other sequences in the *Acanthamoeba* sp. ribosomal DNA database and was determined to be genotype T1, associated with other isolates of *Acanthamoeba* obtained from granulomatous amebic encephalitis infections in humans.

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# 1. Introduction

Several species of the genera *Acanthamoeba*, *Balamuthia*, and *Naegleria* are free-living amoebae that can cause encephalomyelitis in animals and humans (Martinez and Visvesvara, 1997; Visvesvara, 1999). *Acanthamoeba* spp. infections have been reported to cause acute to chronic central nervous system (CNS) infections in

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three dogs (Ayers et al., 1972; Pearce et al., 1985; Bauer et al., 1993; Brofman et al., 2003). We report *Acanthamoeba* sp. infection in a dog and document isolation of the parasite from its brain stored at -70 °C for two months.

## 2. Materials and methods

## 2.1. Naturally-infected dog

An approximately one-year-old Labrador-type male dog was brought to All Pets Veterinary Clinic,

Macomb, Illinois, USA with a complaint of muscle stiffness and apparent discomfort with anorexia. The animal had been obtained as a stray about five months previously, was allowed to run at large, and had no history of vaccination. Subjective signs of discomfort were noted on flexion of the extremities and cervical spine and on palpation of the abdomen, primarily the dorsal right quadrant. Rectal temperature, hematologic and serum enzyme values were all within reference normal ranges. Abdominal and cervical radiographs were unremarkable. Dexamethasone, enrofloxacin, and intravenous lactated ringer's solution were administered. After 24 h the animal was subjectively less stiff, but had developed nystagmus. Oxytetracycline was administered as an intravenous bolus. The following day the dog was in lateral recumbancy and unable to rise, had multiple seizures, and repeatedly pawed at the face. Due to the deteriorating condition the owners elected euthanasia.

#### 2.2. Postmortem examination

A complete necropsy examination was made. Brain, lung, heart, liver, kidney, spleen, mesenteric lymph node, thyroid, thymus, pancreas, multiple sections of intestine, stomach, urinary bladder, and skeletal muscle were collected, fixed in neutral buffered 10% formalin, and routinely processed for histologic examination. Sections were examined after staining with hematoxylin and eosin (H and E). Unfixed tissues were retained at  $-70\,^{\circ}$ C. Brain was examined by flourescent antibody test for rabies virus antigen.

# 2.3. Immunofluorescence for amoeba species

Immunofluorescence tests were conducted by reacting sections of brain with polyclonal anti-Acanthamoeba castellanii, anti-Naegleria fowleri, and anti-Balamuthia mandrillaris antibodies (Visvesvara, 1999).

## 2.4. Isolation and cultivation of amoebae

Attempts were made to isolate the organism from frozen-thawed unfixed brain tissue. The brain had been stored at -70 °C for 60 days and was transferred on dry ice from the laboratory in

Galesburg, Illinois to the Centers for Disease Control (CDC), Atlanta, Georgia, where cultivation was attempted as previously described (Visvesvara, 1999). Approximately 3 g of brain tissue was triturated in about 0.5 ml of amoeba-saline and one-half of the mixture was inoculated onto a monkey kidney cell (E6) monolayer and the other half onto nonnutrient agar coated with Escheria coli. Amoebae grew out of both cultures within a week and were identified as Acanthamoeba sp. based on the characteristic cyst morphology (Visvesvara, 1999). Amoebae growing with bacteria were subsequently washed off of the agar plate and inoculated into Acanthamoeba growth medium containing 100 µg/ml gentamicin and 100 µg/ml imipenem and incubated at 35 °C. After 24 h of incubation the culture medium was removed and replaced with fresh medium. Three such transfers were sufficient to obtain a culture free of bacteria.

## 2.5. Isolation of DNA and PCR

A centrifuged pellet of amoebae was suspended in 200 µl UNSET buffer and DNA extracted (Schroeder et al., 2001; Booton et al., 2002). Subsequently, nuclear 18S rDNA Acanthamoeba genus specific amplicon ASA.S1 was amplified by PCR using genusspecific primers JDP1 (5'-GGCCCAGATCGTTTA-CCGTGAA-3') and JDP2 (5'-TCTCACAAGCTGC-TAGGGAGTCA-3') (Schroeder et al., 2001; Booton et al., 2002). Electrophoresis on a 1% agarose gel produced a product of an expected size of ~450 bp (Fig. 1). The PCR product was then sequenced using an ABI 310 automated fluorescent sequencing system (Applied Biosystems, Foster City, CA) (Schroeder et al., 2001; Booton et al., 2002). The PCR analyses were performed on amoebae cultivated from the brain tissue because all the fresh brain tissue was used up for initiating the culture.

Additionally, mitochondrial 16S rDNA multiplex PCR also was performed to test for the presence of either *Acanthamoeba* spp. or the closely related pathogenic amoeba *Balamuthia mandrillaris* (Booton et al., 2003a). The *Acanthamoeba*-specific PCR amplimer was directly sequenced using primers previously used in our laboratory for sequence analysis of this gene (Ledee et al., 2003).

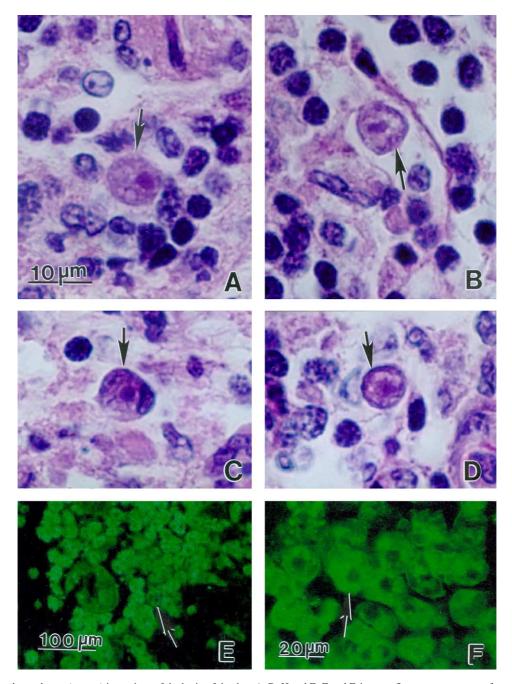


Fig. 1. Acanthamoeba sp. (arrows) in sections of the brain of the dog. A–D, H and E; E and F, immunofluorescence pattern of amoebae in the brain tissue after reaction with anti-Acanthamoeba antibodies. Bar in A applies to parts A–D. Note trophozoites (A–C) with prominent eccentric nucleus, and a cyst (D) with irregular outline of the cyst walls.

## 3. Results and discussion

The carcass was in good condition and body fat stores were normal. There was abundant 4 mm pale foci scattered within the myocardium. The kidneys had multifocal to coalescing, 2–5 mm reddish brown, slightly raised foci primarily in the cortex.

Histologically in the brain stem, cerebellar pedicle, and spinal cord, the meninges and superficial neuropil were infiltrated with macrophages, lymphocytes, and neutrophils. Locally extensive necrosis and spongiosis accompanied by gitter cells were present in the superficial neuropil. Occasional blood vessels were characterized by fibrinoid necrosis and thrombosis. Scattered within the affected areas were round amoebalike protozoa. In H and E-stained sections these protozoa resembled enlarged macrophages. Under high illumination they could be distinguished from macrophages by their eccentric nucleus and a large nucleolus based on optimally stained sections (Fig. 1A-D). A vacuole around the amoebae was also helpful in locating amoebae. Multiple sections of spinal cord demonstrated decreasingly severe lesions distally to about the thoracolumbar junction. The kidney had multifocal to coalescing infiltrates of macrophages, lymphocytes, and neutrophils, with occasional intralesional amoebae. Severe, locally extensive pyogranulomatous infiltrates were also identified in the myocardium, thyroid, lymph node, and pancreas, also containing occasional organisms, occasionally within macrophages. Minimal widely scattered primarily perivascular infiltrates of mononuclear cells were in the pulmonary alveolar walls, but no organisms were detected in H and E stained sections.

Rabies testing was negative. The amoebae in the section treated with anti-A. castellanii antibodies reacted brightly (Fig. 1E and F), but showed no reactivity with the other antisera. The polyclonal antibody is genus but not species specific, and identified the isolate as Acanthamoeba sp.

The source of infection in this case was not identified. The route of entry has been speculated to be oral, nasal, or ocular. In this case, extensive systemic spread was identified, indicative of hematogenous spread. The histological appearance of the lesions suggests concurrent development, and does not point to a specific route of infection. The multicentricity of the infection was striking and consistent with

previously reported cases, although lesions in thyroid have not been reported previously in dogs. Unlike earlier reports, pulmonary lesions were not a prominent feature. Clinical disease progressed rapidly once manifested, a common feature of granulomatous amebic encephalitis in nonhuman species.

Clinical pathology data in this case, however, were not revealing. Fortunately, brain tissue had been frozen and in vitro cultivation and subsequent PCR (Fig. 2) helped to reach a definitive diagnosis. The nuclear 18S rDNA sequence obtained was compared to other sequences in the *Acanthamoeba* ribosomal DNA database and was determined to be genotype T1, which is associated with other isolates of *Acanthamoeba* obtained from granulomatous amebic encephalitis infections (Stothard et al., 1998). Genotype T1 was originally associated with a strain (CDC:V006) that was isolated in 1982 from the brain tissue of a granulomatous encephalitis case.

Next, multiplex PCR targeting the mitochondrial 16S rDNA revealed (Fig. 3) that the culture contained only *Acanthamoeba* as it produced only the *Acanthamoeba*-specific PCR amplimer of ~600 bp (Ledee et al., 2003). Direct sequencing of this product confirmed that it was *Acanthamoeba* sp. Furthermore, the 16S rDNA sequence was identical to other *Acanthamoeba*. sp. genotype T1 isolates, the same

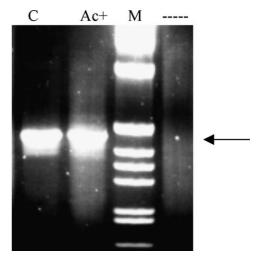


Fig. 2. Acanthamoeba genus-specific nuclear 18S rDNA PCR. Legend: arrow indicates position of Acanthamoeba specific PCR amplimer; C, canine isolate PCR; Ac+, Acanthamoeba positive control; M = 1 kbp marker; (- - -) negative control is template-free master mix.

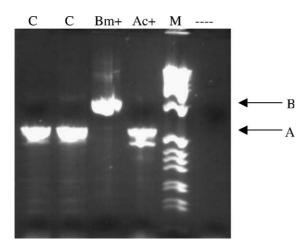


Fig. 3. Multiplex 16S mitochondrial rDNA PCR. Legend: Arrow B, *Balamuthia mandrillaris* 16S genus-specific amplimer; arrow A, *Acanthamoeba* sp. 16S genus-specific amplimer; C, canine isolate multiplex PCR; Bm+, *Balamuthia mandrillaris* positive control; Ac+, *Acanthamoeba* sp. positive control; M = 1 kbp marker; (- - -) negative control is template-free master mix. *Acanthamoeba* genus specific nuclear 18S rDNA PCR.

genotypic conclusion provided by the nuclear 18S rDNA analysis. If *Balamuthia* were present a second PCR product of 1075 bp would have been produced by the *Balamuthia*-specific primer set used in the multiplex PCR, however, no *Balamuthia*-specific band was observed (Booton et al., 2003a, 2003b). Therefore, the presence of *Acanthamoeba*-specific amplimers in both nuclear and mitochondrial rDNA analyses lead to the conclusion that the infection of this dog was due to *Acanthamoeba*, specifically genotype T1. Lastly, multiplex analysis indicates that no *Balamuthia* infection was present in this canine.

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